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Expression of the *Agrobacterium rhizogenes rol*C Gene in a Deciduous Forest Tree Alters Growth and Development and Leads to Stem Fasciation[1]

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We have altered the growth and development of a deciduous forest tree by transforming hybrid aspen (*Populus tremula* × *Populus tremuloides*) with the *Agrobacterium rhizogenes rol*C gene expressed under the strong cauliflower mosaic virus 35S promoter. We demonstrate that the genetically manipulated perennial plants, after a period of dormancy, maintain the induced phenotypical changes during the second growing period. Furthermore, mass-spectrometrical quantifications of the free and conjugated forms of indole-3-acetic acid and cytokinins and several gibberellins on one transgenic line correlate the induced developmental alterations such as stem fasciation to changes in plant hormone metabolism. We also show that the presence of the RolC protein increases the levels of the free cytokinins, but not by a process involving hydrolysis of the inactive cytokinin conjugates.

Genetic engineering of annual plant species has proven to be a valuable technique both for commercial applications and to manipulate and to study the different aspects of plant biology. With the recent development of new transformation protocols, these techniques now can be extended to commercially and biologically interesting perennials such as fruit and forest trees to alter the quality and yield parameters and also to learn more about how growth and development are regulated in woody plants. However, although these techniques are available and the list of transgenic tree species is constantly growing (reviewed by Jouanin et al., 1993), there are still only a few reports available on the regeneration of phenotypically altered trees due to the controlled, ectopic expression of a heterologous gene (Weigel and Nilsson, 1995; Tuominen et al., 1995).

We transformed hybrid aspen (*Populus tremula* × *Populus tremuloides*), a temperate-zone deciduous tree species, with a gene that has been demonstrated to drastically alter the growth and development of annual plants: the *rol*C gene from the plant pathogenic bacterium *Agrobacterium rhizogenes*. During the normal bacterial infection process, several *rol* genes are transferred to and expressed in the plant cell. The *rol*A, *rol*B, and *rol*C genes have a central role in this process, since they are necessary for the production of hairy roots, a massive formation of adventitious roots, which is the outcome of the infection (White et al., 1985). The molecular function of the encoded *rol* gene product is still unknown, but it is generally considered to be different from that of the oncogenic genes located on the *Agrobacterium tumefaciens* T-DNA, which encodes enzymes involved in the biosynthesis of the plant hormones IAA and cytokinin (see Gaudin et al., 1994, for review).

To date, gene constructs that contain the single *rol*C gene expressed from the cauliflower mosaic virus 35S promoter have been used to transform species such as tobacco (*Nicotiana tabacum*) (Schmülling et al., 1988; Nilsson et al., 1993), potato (*Solanum tuberosum*) (Fladung, 1990), and *Atropa belladonna* (Kurioka et al., 1992). The growth and development of the transformants have been drastically altered. However, the plants always display variations of a common phenotype: they are dwarfed and have an increased number of small, light-green leaves, short internodes, and reduced apical dominance. Some of these phenotypic alterations suggest that the effects of *rol*C expression might be linked to an increase in cytokinin activity (Schmülling et al., 1988). It has been proposed that RolC is a β-glucosidase that is capable of releasing free active cytokinins from their inactive glucosidic conjugates (Estruch et al., 1991). However, this hypothesis was not supported by the subsequent quantifications of either the substrates or the immediate products of the proposed reaction in vivo in transgenic *rol*C-expressing plants. Later quantifications in 35S::*rol*C tobacco failed to detect any significant increase in free cytokinins (Nilsson et al., 1993; Schmülling et al., 1993) or decrease in cytokinin conjugates (Nilsson et al., 1993). In 35S::*rol*C potato, however, an extreme phenotypical alteration was observed together with a general increase in free

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Abbreviations: DHZ, dihydrozeatin; iP, isopentenyl adenine; iPA, isopentenyl adenosine; LC, liquid chromatography; MeOH, methanol; SIM, selected ion monitoring; wt, wild type; Z, zeatin; ZOG, zeatin *O*-glucoside; ZR, zeatin 9-riboside; ZROG, zeatin riboside *O*-glucoside; Z9G, zeatin 9-glucoside.

cytokinins, but no cytokinin conjugates were quantified (Schmülling et al., 1993).

To date, all 35S::*rol*C plants that have been described in the literature belong to the Solanaceae. To test the generality of the *rol*C-induced effects on plant phenotype and hormone metabolism, it is enlightening to study the effects of *rol*C expression on a completely unrelated species with different growth characteristics, such as a forest tree. Under natural conditions the *A. rhizogenes*-induced hairy-root disease has been found in only three species, which are all woody perennials: apple (*Malus sylvestris* L.) and two species of *Spiraea* (De Cleene and De Ley, 1981). This fact makes tree species an obvious choice for studying the effects of *rol* gene expressions, since they are closer to the natural hosts than are solanaceous plants.

Here we show that the expression of the *A. rhizogenes* *rol*C gene in a deciduous hardwood tree species has a pronounced effect on growth and development in a RolC dose-dependent manner. In the highest-expressing plants, these changes included the induction of stem fasciation, a phenotypical alteration that has not been previously associated with *rol*C expression. Furthermore, the expression of the *rol*C gene and the associated phenotypical changes, after a period of dormancy, were shown to be maintained during the second growing period. The maintenance of a phenotype over time is important if transgenic trees are to be useful in a more applied sense. Finally, in an extensive quantification of plant hormones in the most severely affected line, using high-resolution GC- and LC-MS techniques, including internal standards for the compounds measured, we demonstrate that some of these alterations can be correlated to major changes in hormone metabolism, especially in cytokinins and gibberellins, which partly confirm and significantly extend earlier data that were obtained with tobacco and potato. Our results provide further insight into the relationship between plant hormone metabolism and the regulation of growth and development and the possible functions of the RolC protein.

MATERIALS AND METHODS

Gene Constructs and Plant Transformation

The construction of pPCV702-*rol*C was described previously (Nilsson et al., 1993). This construct contains the *Agrobacterium rhizogenes rol*C gene under the control of the cauliflower mosaic virus 35S RNA promoter. The pPCV702-*rol*C and pPCV702 (control) constructs were transferred to *Agrobacterium tumefaciens* and used to transform hybrid aspen (*Populus tremula* × *Populus tremuloides*, clone T89) stem segments as described previously (Nilsson et al., 1992). Thirty independent pPCV702-*rol*C and 10 pPCV702 transformants, as well as 30 T89 wt plants, were regenerated as described previously (Nilsson et al., 1992). Subsequently, one of the pPCV702-*rol*C plants with the highest level of *rol*C expression, as determined by northern hybridization, was surface-sterilized with mercury chloride and multiplied in tissue culture. This clone was regenerated again and used for a more detailed, phenotypical characterization, as well as for hormone measurements.

Plant Cultivation

After selection and shoot and root initiation, transgenic and wt T89 plants were potted in mineral wool (Esskron, Malmö, Sweden) and cultivated in a controlled-environment chamber having a day/night temperature of 20/10°C, an 18-h light/6-h dark photoperiod, a photon flux density of 240 μmol m^{-2} s^{-1} from metal halogen lamps (HQI-TS 400 W/DH, Osram, Munich, Germany), and a RH of about 70%. The plants were watered as required with a complete nutrient solution (Ingestad, 1970) containing 100 mg nitrogen L^{-1}. To induce dormancy, the plants were subjected to a combination of short photoperiods (8 h of light and 16 h of dark) and warm temperatures (20/10°C, day/night) for 6 weeks to induce bud set. Thereafter, the temperature during the photoperiod was reduced to 15°C for an additional 10 weeks; by the end of this period, leaf abscission had occurred. The plants were then transferred to a cold room with low day/night temperature of 4/4°C and the same short photoperiod to satisfy the chilling requirement of the apical and cambial meristems. After 2 months the plants were transferred back to the original long-day conditions for meristem reactivation.

Sample Preparation

Rooted in vitro-amplified individual plants, originating from the *rol*C transformant showing the most severe phenotype, as well as plants of wt origin, were planted in mineral wool and allowed to grow for 6 weeks before sampling. At this time, the wt plants were about 85 cm tall, whereas the *rol*C-expressing plants were about 40 cm tall. At this stage, both wt and *rol*C plants were in a steady state of growth. The plants were cut at one-half of their total height, and the upper leaves and upper internodes were sampled. All samples were immediately frozen in liquid nitrogen and freeze-dried and then homogenized to a fine powder in liquid nitrogen using a coffee grinder and stored at −70°C until analysis.

Two months after reactivation, samples of the shoot apical region were taken from plants in their second growing period. The distal 5 mm, including the youngest, expanding leaves of both the main and side shoots, were pooled and prepared as described above.

Northern Blot and Hybridization

For RNA isolation, tissue samples were taken from the leaves of seven independent, primary regenerants that showed an increasingly pronounced RolC phenotype. Samples from the in vitro-propagated *rol*C line were also taken, as specified above. Total RNA was isolated and used for northern blot and hybridizations with *rol*C as a probe, as described earlier (Nilsson et al., 1993). The amount of RNA that was analyzed was checked before loading onto gels by UV-spectrophotometric quantification and after gel electrophoresis and capillary transfer by UV visualization of ethidium bromide-stained RNA on gels and filters.

Quantification of IAA

Quantitative analysis of IAA and IAA conjugates (liberated after hydrolysis for 1 h at 100°C in 7 M NaOH) was

performed by GC-SIM-MS using [$^{13}C_6$]IAA (Cambridge Isotope Laboratories, Woburn, MA) as an internal standard. The method for purification and quantitative analysis by GC-MS was previously described by Sundberg (1990) and Sitbon et al. (1992).

Quantification of GAs

Freeze-dried samples corresponding to 5 g of fresh weight were extracted overnight in 50 mL of 80% methanol. The following deuterated GAs (10 ng) were added as internal standards: 17,17-[$^2H_2$]GA_1, 17,17-[$^2H_2$]GA_{19}, 17,17-[$^2H_2$]GA_{20}, and 17,17-[$^2H_2$]GA_8 (Lewis Mander, Australian National University, Canberra, Australia). After concentration in vacuo, the sample was diluted with 5 mL of 100 mM potassium phosphate buffer (pH 8.0) and applied to a column containing 10 mL of insoluble poly-*N*-vinylpolypyrrolidone that was pre-equilibrated with 100 mL of the same buffer. The column was rinsed with 50 mL of buffer, which was collected. The pH of the eluate was adjusted to 2.8 and was partitioned against ethyl acetate followed by extractions on aminopropyl columns as described by Moritz and Monteiro (1994). The samples were further purified, and the respective GA fractions were separated by reversed-phase HPLC as described by Nilsson et al. (1993). The HPLC fractions were methylated with ethereal diazomethane and analyzed by GC-SIM-MS as described previously (Olsen et al., 1995).

Quantification of Cytokinins

Freeze-dried samples that corresponded to 10 g of fresh weight were extracted in 100 mL of 80% methanol/50 mM Tris-HCl (pH 7.5), which contained 0.02% sodium diethyldithiocarbamate as an antioxidant. The following deuterated cytokinins were added as internal standards: 50 ng of [$^2H_5$]Z, [$^2H_3$]DHZ, and [$^2H_6$]iP, and 100 ng of [$^2H_5$]ZR, [$^2H_5$]Z7G, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_3$]DHZR, and [$^2H_6$]iPA (Apex Organics, Honiton, UK). Furthermore, tritiated standards were added as fractionation and recovery markers during the purification procedure. These included 50,000 dpm of [^{3}H]Z, [^{3}H]ZR, [^{3}H]iP, and [^{3}H]iPA (0.9–1.65 TBq/mmol). After concentration in vacuo, the sample was diluted with 10 mL of 50 mM Tris-HCl (pH 7.5) and treated with 0.2 units of alkaline phosphatase (P-5931, Sigma).

Initial purification was carried out by applying the sample onto a column that contained 10 mL of insoluble poly-*N*-vinylpolypyrrolidone equilibrated with 50 mM Tris-HCl (pH 7.5). The column was rinsed with 50 mL of buffer, which was collected. The eluate was adjusted to pH 8.2 and partitioned five times against water-saturated (pH 8.2) 1-butanol. The combined butanol fractions were evaporated to dryness and dissolved in 10 mL of 50 mM Tris-HCl (pH 7.5). Further purification was carried out by anion-exchange columns combined with C_{18} columns, and cation-exchange columns, essentially as described (Nilsson et al., 1993).

Final purification and fractionation of the individual cytokinins were performed on a reversed-phase HPLC system as described previously (Nilsson et al., 1993). The recovery was 35 to 80%, except for iP, for which it was less than 20%. Together with the low sensitivity of iP in the MS analysis, this led to undetectable levels of iP in the subsequent quantifications. The HPLC fractions of interest were reduced to dryness and acetylated in 20 μL of pyridine and 20 μL of acetic anhydride prior to analysis. After heating at 80°C for 2 h, the samples were evaporated to dryness and dissolved in H_2O/MeOH (95:5, v/v) and further analyzed by the capillary frit-fast atom bombardment LC-MS system (JEOL), as described previously by Imbault et al. (1992). In all cases the mobile phase consisted of 1% acetic acid, 1% glycerol, and 0.03% triethylamine, with 45% MeOH for the quantification of Z and DHZ; 55% MeOH for ZOG; 60% MeOH for ZR, DHZR, iP, and iPA; and 68% MeOH for ZROG. Ions were generated with a beam of 6-kV Xe atoms at an emission current of 20 mA and accelerated with 10 kV. Samples were analyzed at a resolution of 5,000 to 10,000 by SIM using accelerating voltage switching and glycerol as a reference compound, except for the glucosides that were analyzed by selected reaction monitoring recording specific reactions at a low resolution. The dwell time was 300 ms. Calibration curves for all cytokinins were recorded, and all data were processed by a JEOL data system (MS-MP7010D).

Histological Sections

Shoot apical regions and the youngest, fully expanded leaves of wt and *rol*C-expressing plants were fixed for at least 3 d in 3% glutaraldehyde, 25 mM sodium phosphate buffer, pH 6.8, dehydrated in an ascending ethanol series, and embedded in Historesin (Reichert-Jung, Heidelberg, Germany). Transverse and longitudinal leaf sections were obtained at 2 to 3 μm with a rotary microtome. The sections were stained with toluidine blue, mounted in Entellan (Merck, Darmstadt, Germany), and examined under a light microscope (Axioplan, Zeiss).

The size of palisade cells from the leaves was measured under the microscope by a measuring ocular. About 150 cells from a total of eight different 35S::*rol*C and wt plants were determined, and the thickness of transgenic and wt leaves was determined.

RESULTS

Generation of *rol*C-Expressing Hybrid Aspen

The regeneration of *rol*C-transformed plants was normal, i.e. there was no reduction in either the production of shoots from the stem segments transformed with the vector pPCV702-*rol*C or the subsequent rooting of these shoots, compared with the control plants transformed with the vector pPCV702 alone or with wt plants that had been regenerated in vitro. Thirty independently regenerated, kanamycin-resistant plants transformed with the vector pPCV702-*rol*C, 10 pPCV702-transformed control plants, and 30 regenerated wt T89 plants were potted in mineral wool and transferred to controlled-environment conditions in a growth chamber. The pPCV702-transformed plants

were phenotypically indistinguishable from wt plants, but the *rol*C-expressing plants displayed varying degrees of the characteristic phenotype shown in its most extreme form (Fig. 1A, right). Typical features of the *rol*C-expressing plants included stunted growth with an increased number of small, light-green leaves and short internodes. The severity of these alterations were always correlated to each other. In addition, five of the most affected individual transformants also displayed stem fasciation, which corresponded to about 15% of all plants (see below). During the last 4 years we have regenerated several thousand hybrid aspen plants under similar conditions (Nilsson et al., 1992; Nilsson and Weigel, 1995; Tuominen et al., 1995; O. Olsson, unpublished data), but we have never seen stem fasciation in plants other than the *rol*C-expressing ones. Northern analysis of the different regenerants revealed various degrees of expression of the *rol*C transcript (Fig. 2A). The severity of the resulting phenotype correlated with the magnitude of expression of *rol*C (data not shown) and indicated that a high level of *rol*C expression was necessary to provoke the phenotypic changes.

Phenotypic Characterization

One of the plants with the highest levels of expression (Fig. 1A, right) was multiplied in a tissue culture and regenerated again. The resulting line was chosen for a closer examination of the alterations in development and hormone metabolism; a representative plant is shown in Figure 1, B, D, and F. Early in their development, these plants displayed a reduced apical dominance, which produced more side shoots than wt (not shown). However, if these side shoots were removed, growth subsequently was characterized by a single shoot with normal apical dominance (Fig. 1B). A similar growth response was earlier reported for 35S::*rol*C tobacco cv W38 (Nilsson et al., 1993). Furthermore, the *rol*C-expressing hybrid aspen plants grew slower than wt (*rol*C approximately 14 mm/day, wt approximately 24 mm/day) and produced more leaves (*rol*C approximately 3.3 leaves/day, wt approximately 1.0 leaves/day), often with an altered phyllotaxis.

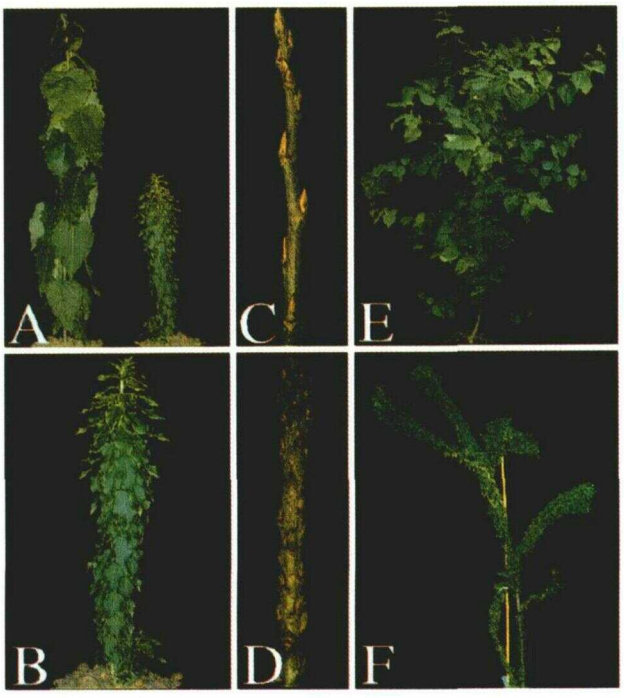

Figure 1. Phenotypes of wt (A, C, and E) and 35S::*rol*C (B, D, and F) hybrid aspen plants. A and B, Six-week-old wt (A, left) and *rol*C plants in their first growing period. C and D, Dormant shoots after 2 months at 4°C. E and F, Plants in their second growing period, 2 months after reactivation.

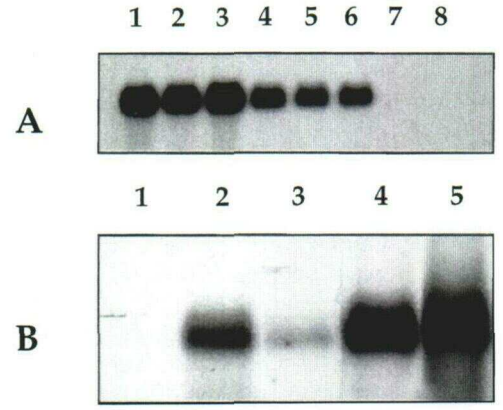

Figure 2. Northern analysis of the transcription of the *rol*C gene. A, Samples from the young leaves of individual pPCV702-*rol*C transformants (lanes 1–7) and a representative pPCV702-transformed control plant (lane 8). Lanes 1 to 7 represent plants with a gradually decreasing *rol*C phenotype, where lane 1 is the 35S::*rol*C plant seen in Figure 1A, right, lane 6 displays a very weak phenotype, and lanes 7 and 8 are indistinguishable from wt. B, Samples from representative pPCV702-transformed control and 35S::*rol*C plants used for hormone quantifications. Lane 1, wt upper leaves. Lane 2, *rol*C upper leaves. Lane 3, *rol*C upper internodes. Lane 4, *rol*C nonfasciated shoot apical regions. Lane 5, *rol*C fasciated shoot apical regions. Total RNA (25 μg) was loaded in each lane. The *rol*C transcript was compared to a DNA standard run in parallel and was estimated to be about 600 bases.

In a manner similar to that of several of the primary transformants, this 35S::*rol*C hybrid aspen clone displayed new phenotypic alterations not previously associated with *rol*C expression. After reaching a height of approximately 60 cm, most of the transgenic plants started to fasciate. The shoot apices enlarged, sometimes so much that the meristem became exposed and was not covered by the youngest leaves (Fig. 3, A–C). Furthermore, the meristem expanded in one plane and lost its radial symmetry, which resulted in the formation of a ridge-like meristem that produced a flat stem (Fig. 3, D and E). In some cases the growth of fasciated stems resulted in spiralization (Fig. 3F) and bifurcation (not shown). Histological examinations on the transverse sections of fasciated stems displayed a single fused ring of xylem (Fig. 4A), which revealed that a true fasciation had occurred, derived from a single apex, in contrast to a fasciation that it was derived from the fusion of several apices (Gorter, 1965). Furthermore, longitudinal sections of fasciated apices in the plane of the fasciations showed that a dramatically enlarged and flattened meristem was pro-

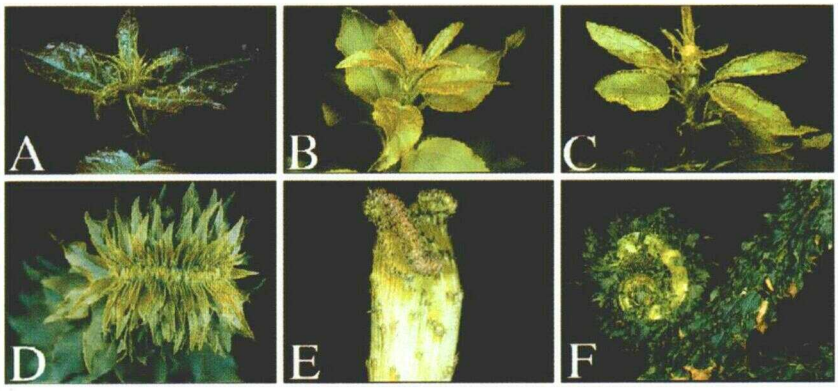

Figure 3. Phenotypes of shoot apical regions from wt (A) and 35S::*rolC* (B–F) plants. B, Before fasciation; C, early fasciation; D, late fasciation; E, dormant-fasciated shoot; F, spiralization.

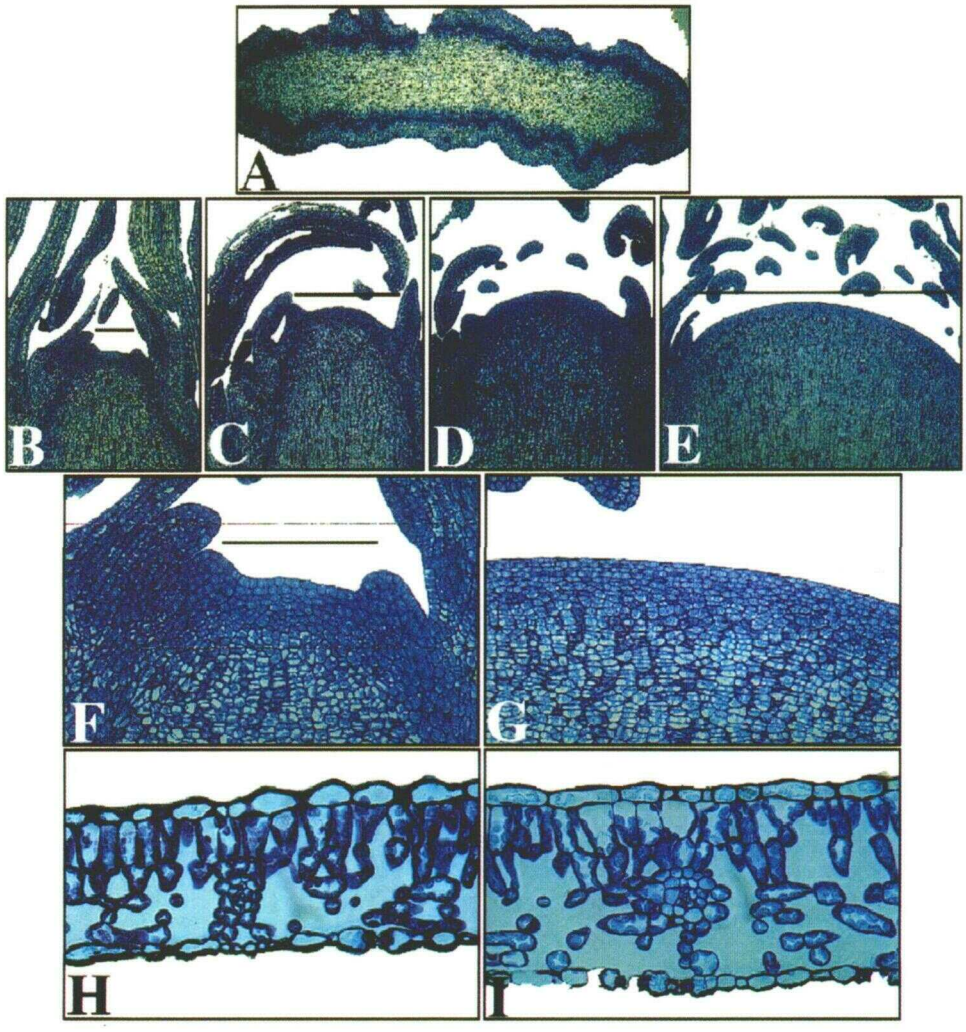

Figure 4. Histological sections from shoot apices and leaves of wt and 35S::*rolC* plants. A, Transverse section through a fasciated *rolC* stem. The stem displays a single fused ring of xylem. B to G, Median sections through shoot apices of wt (B and F) and *rolC* plants (C, D, E, and G). C, *rolC* apex before any signs of fasciation. D, Early stage of fasciation. E, Later stage of fasciation. F, Close-up of wt apex. G, Close-up of *rolC* apex; note the increased number of smaller cells. H and I, Transverse sections of the youngest, fully expanded leaves of wt (H) and *rolC* (I) plants. Size bars are included in B (250 μm), C (600 μm), E (1950 μm), and F (250 μm) to facilitate comparison. The total width of A is 5350 μm, the thickness of H is 92 μm, and the thickness of I is 138 μm. F and G are at the same magnification.

duced, which initiated leaf primordia on its flanks (Fig. 4, C–E). Fasciated apices contained more and smaller cells than wt (Fig. 4, F and G). It is interesting that longitudinal sections of apices from the *rol*C-expressing plants that showed no sign of fasciation also revealed the presence of significantly enlarged meristems composed of an increased number of smaller cells (Fig. 4, compare B and C).

The leaves of *rol*C-expressing plants were much smaller than wt leaves (Fig. 1A). Transverse sections of the youngest, fully expanded leaves of wt and *rol*C plants showed that, surprisingly, this was not attributable to smaller cells in the 35S::*rol*C plants. On the contrary, leaves from the *rol*C plants appeared thicker and were composed of larger palisade cells than the wt leaves (Fig. 4, H and I). Direct cell measurements on eight different leaves from wt and *rol*C plants confirmed this observation (wt, 92.05 ± 0.65 μm [SD], n = 150; *rol*C, 137.8 ± 1.05 μm [SD], n = 150). Furthermore, average cell height and cell width in the transverse sections were 27.29 ± 0.24 μm (SD), n = 150 and 15.65 ± 0.15 μm (SD), n = 150, respectively, in wt but had increased to 33.83 ± 0.32 μm (SD), n = 150 and 20.30 ± 0.40 μm (SD), n = 150, respectively, in the *rol*C plants.

The phenotype of the dormant shoots reflected the growth alterations observed previously. Nonfasciated *rol*C shoots displayed a dramatically increased number of closely positioned buds, which reflected the positions of the leaves on the growing plant (Fig. 1, C and D). Fasciated shoots exhibited the flattened stem and the ridge-like apical meristem (Fig. 3E).

The phenotype of wt hybrid aspen plants during their second year of growth is characterized by the presence of lateral branches (Fig. 1E). However, the *rol*C-expressing plants lacked such branches (Fig. 1F), and instead, the continued growth of the flattened stems resulted in an even more dramatic phenotype than during their first year of growth. This phenotype continued to occur after two additional cycles of growth and dormancy (data not shown).

Northern Analysis of Transformed Plants

Northern-analysis experiments revealed that the primary transformants that displayed the most pronounced phenotypical alterations showed the highest levels of *rol*C expression (Fig. 2A). More detailed northern experiments also were performed on the amplified, high *rol*C-expressing line on the same tissue samples that were used in subsequent hormone quantifications. These plants displayed a strong expression in young leaves, a relatively weaker expression in the upper internodes, and the strongest expression in the apical regions (Fig. 2B). This expression pattern was similar to that described earlier for the 35S promoter in hybrid aspen (Nilsson et al., 1992), which showed that the selection for a different expression pattern did not occur during the regeneration procedure. The data also indicate that *rol*C was strongly expressed in all parts of the plants used for hormone quantifications.

Quantification of IAA

Compared with wt plants, the concentration of free IAA in *rol*C-expressing plants was significantly reduced in both the upper leaves and the apical region (Fig. 5A), whereas the content of conjugated IAA was not altered in the upper leaves and was only slightly increased in the apex (Fig. 5B). This suggests that the reduced free IAA concentration in 35S::*rol*C plants was not due to increased conjugation. The levels of free and conjugated IAA did not differ between fasciated and nonfasciated apical regions of *rol*C transformants.

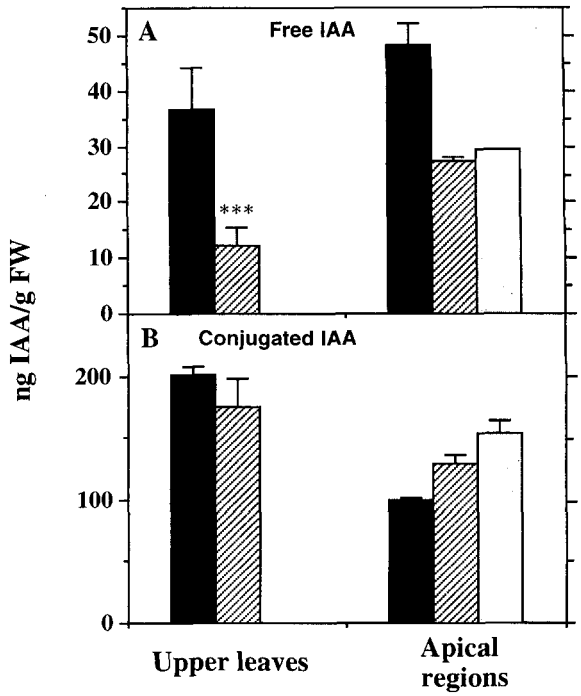

Figure 5. Content of free and conjugated IAA as determined by GC-MS. A, Content of free IAA. B, Content of conjugated IAA. IAA was quantified in the upper leaves of wt (solid bars) and *rol*C (hatched bars) hybrid aspen plants in their first period of growth (mean ± SE, n = 5) and in apical shoot regions pooled in 2 groups of 5 plants from 10 wt and 10 *rol*C plants in their second period of growth (mean ± range). Nonfasciated (hatched bars) and fasciated (shaded bars) *rol*C shoots were sampled separately. The pooling of apical regions did not allow statistical analysis. Note the different scales for IAA content. ***, Significantly different at $P < 0.001$ (t test). FW, Fresh weight.

Quantification of Gibberellins

The concentrations of the active GA_1 and its immediate precursor GA_{20} were significantly lower in the upper internodes, the upper leaves, and the apical regions of *rol*C plants, compared with wt plants (Fig. 6, A–C). It is interesting that this was not accompanied by a concomitant decrease in GA_{19}, the precursor of GA_{20} (Fig. 6, A–C), and instead the levels of GA_{19} were doubled in the apical regions of the *rol*C-expressing plants (Fig. 6C). Furthermore, since the levels of the GA_1 deactivation product GA_8 were decreased (Fig. 6, A–C), increased 2β-hydroxylation to form this compound could not be the reason for the decreased levels of GA_1 and GA_{20}. No significant differences were found in the content of different GAs in fasci-

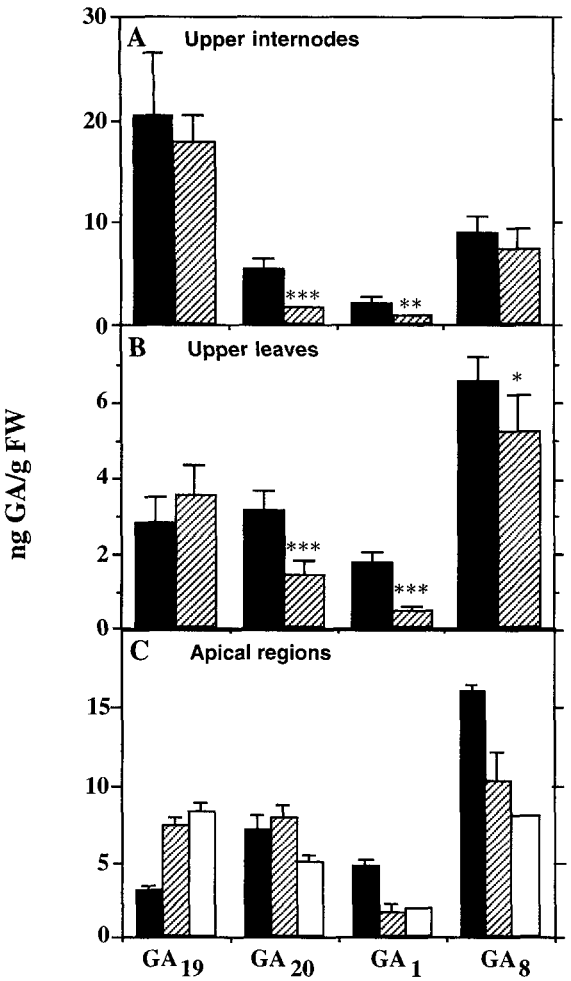

Figure 6. Content of GAs as determined by GC-MS. A, Upper internodes. B, Upper leaves. C, Apical shoot regions. GAs were quantified in the upper leaves and internodes of wt (solid bars) and *rolC* (hatched bars) hybrid aspen plants in their first period of growth (mean ± SE, $n = 5$) and in apical shoot regions pooled in 2 groups of 5 plants from 10 wt and 10 *rolC* plants in their second period of growth (mean ± range). Nonfasciated (hatched bars) and fasciated (shaded bars) *rolC* shoots were sampled separately. ***, Significantly different at $P < 0.001$ (*t* test); **, significantly different at $P < 0.01$; *, significantly different at $P < 0.05$. The pooling of apical regions did not allow statistical analysis. Note the different scales for GA content. FW, Fresh weight.

ated compared with nonfasciated *rolC*-expressing apical regions (Fig. 6C).

Quantification of Cytokinins

The upper leaves of the *rolC*-expressing plants contained significantly increased levels of ZR compared with wt plants, whereas the levels of iPA were significantly decreased (Fig. 7B). DHZR was below the level of detection (<0.1 pmol/g fresh weight). The concentrations of the bases DHZ and Z were unaltered or slightly increased (Z, 92% probability level). The levels of the *N*-glucosides Z7G and Z9G were below the level of detection in both wt and *rolC* plants (<0.1 pmol/g fresh weight), whereas the levels of the *O*-glucosides were much higher. The *rolC*-expressing plants contained significantly higher concentrations of ZOG and ZROG than wt plants (Fig. 7B), which indicates that a higher synthesis of Z-family cytokinins was partly overcome by increased conjugation.

In the apical regions of wt and nonfasciated *rolC* shoots, we could detect only three cytokinin species. This was mainly due to tissue-specific substances that interfered with the mass-spectrometrical detection. It is interesting that ZR was dramatically increased in the *rolC*-expressing apical regions to more than 800% of the level in wt (Fig. 7A). A notable increase was also found in the concentration of the *O*-glucoside ZOG, which again suggested an increased synthesis of Z-family cytokinins.

DISCUSSION

The only examples of a forest tree species in which growth and development have been severely altered due to the controlled expression of transgenes are the *rolC*-expressing hybrid aspens described in this paper and the two recent reports that describe the expression of the *A. tumefaciens* T-DNA genes *iaaM* and *iaaH* and the expression

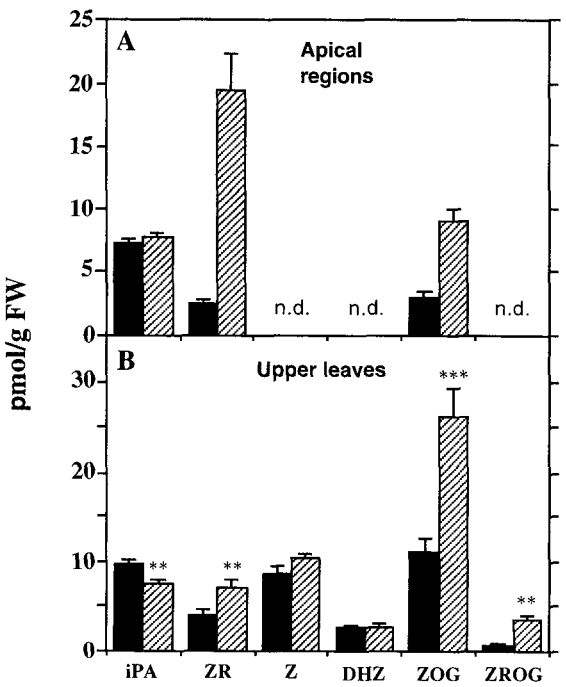

Figure 7. Content of cytokinins as determined by LC-MS. A, Apical shoot regions. B, Upper leaves. Cytokinins were quantified in the upper leaves of wt (solid bars) and *rolC* (hatched bars) hybrid aspen plants in their first period of growth (mean ± SE, $n = 5$) and in apical shoot regions pooled in 2 groups of 5 plants from 10 wt and 10 *rolC* plants in their second period of growth (mean ± range). Fasciated and nonfasciated *rolC* shoots were sampled separately, but cytokinins could only be quantified in nonfasciated shoots. ***, Significantly different at $P < 0.001$ (*t* test); **, significantly different at $P < 0.01$. The pooling of apical regions did not allow statistical analysis. Note the different scales for cytokinin content and that the nucleoside quantifications represent the sum of nucleosides and nucleotides. FW, Fresh weight. n.d., Not detected.

of the *Arabidopsis thaliana LEAFY* gene (Tuominen et al., 1995; Weigel and Nilsson, 1995). Furthermore, the use of a perennial species has enabled us to study the expression of a transgene after a period of dormancy during the second growing season. Here we show that the phenotypic alterations caused by *rol*C expression was maintained stably during the first annual cycle of growth and dormancy, and that there was no sign of inactivation of *rol*C expression during the second growing season. These findings are important for the future application of genetic engineering to forest and fruit tree species.

Phenotypic effects of *rol*C expression from the 35S promoter were previously studied in species belonging to the Solanaceae (Schmülling et al., 1988; Fladung, 1990; Kurioka et al., 1992; Nilsson et al., 1993). The effects observed were similar to those in 35S::*rol*C hybrid aspen plants, a taxonomically distant plant. Transformants are stunted, they have an increased number of small, light-green leaves, and they display a reduced apical dominance during some phase of their development. These phenotypes reveal that the effects of *rol*C expression are general, and that RolC must interfere with the basic processes that are conserved among plant species. This interference seems to be proportional to the amount of RolC protein present, which perhaps indicates a competitive inhibition or a gradual stimulation of another biological process.

However, the stem fasciation described here has not been associated previously with *rol*C expression. It can either be attributed to the use of hybrid aspen as a model system or to the fact that a very high *rol*C expression level has to be reached in order to reach a critical threshold value of RolC, which triggers the developmental process that leads to fasciation. Indeed, a high *rol*C expression could be detected in the shoot apices of fasciated stems (Fig. 2B, lane 5).

In Arabidopsis a number of recessive mutations giving rise to fasciations have been reported. These include *clavata1* (*cla1*) (Leyser and Furner, 1992; Clark et al., 1993), *fasciata1*, *fasciata2* (*fas1*, *fas2*) (Leyser and Furner, 1992), and *fully fasciated* (*fuf*) (Medford et al., 1992). Plants homozygous for these mutations are characterized by the early enlargement of the apical meristem, which divides predominantly in one plane, giving rise to a meristem in the form of a line instead of a point. This meristem then forms a flattened, band-shaped, sometimes bifurcated stem with an altered leaf phyllotaxis. It is interesting that all of these features are observed also in fasciated *rol*C-expressing hybrid aspen plants. For example, the fasciated meristem of *rol*C plants is remarkably similar to that of *clv1* mutants (Clark et al., 1993). The *rol*C-expressing hybrid aspen plants, therefore, represent simple gain-of-function transformants, in which expression of a single gene results in the same fasciated phenotype as the previously described loss-of-function mutations in Arabidopsis.

The shoot apical meristem of 35S::*rol*C plants is both wider and taller than its wt counterpart and contains significantly more cells, even before any sign of fasciation can be observed (Fig. 4). According to the field theory of leaf primordium initiation, the position of a new leaf is determined by the interaction of gradually decreasing fields of substances, inhibitory to leaf initiation, which are produced by the recently initiated leaf primordia (Wardlaw, 1949). These fields might arise from the synthesis and transport of growth regulators, and one of the candidates is IAA. The application of auxins or IAA transport inhibitors alters leaf phyllotaxis and promotes stem fasciation (reviewed by Gorter, 1965). It is interesting that the apical regions of the *rol*C-expressing plants contain significantly lower concentrations of IAA (Fig. 5A). If this is caused by a lower level of synthesis of IAA in the leaf primordia, and IAA is creating the inhibitory fields mentioned above, then these fields would be smaller, which would explain the increased leaf initiation and altered phyllotaxis in *rol*C plants. The increased size of the apical meristem in *rol*C-expressing plants would probably lead also to a larger organ number, since it would provide more room for the initiation of leaf primordia. This has already been suggested for the Arabidopsis mutant *fuf* (Medford et al., 1992), which also displays an increased leaf initiation, but no hormones were measured in that investigation.

The greatest change in the hormone content of *rol*C-expressing apical regions, however, was not the reduced IAA level, but rather the 8-fold increase in the cytokinin ZR (Fig. 7A). This decreased the IAA/ZR ratio from 115 in wt to 8.2 in 35S::*rol*C apical regions. Cytokinins are well-known promoters of cell division, and the high levels of Z-family compounds could therefore explain why the *rol*C apices are larger with an increased number of small cells. In addition, the "fasciation disease," which is caused by the phytopathogen *Rhodococcus fascians*, has recently been shown to depend on the presence of an isopentenyl transferase (*ipt*) gene carried by the bacterium (Crespi et al., 1992). This gene is involved in the production of cytokinins that are excreted from the bacterium. Furthermore, the symptoms of the *R. fascians* disease can be duplicated closely by treatment with the synthetic cytokinin kinetin (Thimann and Sachs, 1966). These observations give further support to the involvement of cytokinins in the induction of fasciation.

The hormone quantifications show that the 35S::*rol*C plants have a significantly altered metabolism of all the investigated hormones. This is not surprising, considering the dramatically altered phenotype of the *rol*C-expressing plants, which in addition makes it difficult to distinguish between the primary and secondary effects of the *rol*C activity.

The reduced levels of free and conjugated IAA in *rol*C-expressing hybrid aspen plants (Fig. 5) indicate a lowered IAA biosynthesis. This is similar to 35S::*rol*C tobacco, in which a reduced amount of IAA conjugates was found (Nilsson et al., 1993). However, in that system this was probably a secondary effect, since an IAA-overproducing 35S::*iaaM*/35S::*iaaH* tobacco plant, crossed with a 35S::*rol*C plant, displayed a complete mixture of the respective phenotypes (O. Nilsson, T. Moritz, B. Sundberg, G. Sandberg, and O. Olsson, unpublished data).

The results of the GA quantifications (Fig. 6) indicate that *rol*C expression interferes with the biosynthesis of the active GA_1, since a partial block in the synthesis between the

precursors GA_{19} and GA_{20} can be detected (Fig. 6). This supports earlier data that were obtained with 35S::*rol*C tobacco leaves, which also showed that increased levels of GA_{19} were linked to lower concentrations of GA_{20} and GA_1 (Nilsson et al., 1993). Here we also quantified the GA_1 deactivation product GA_8 and show that the *rol*C-expressing plants contained reduced levels of this compound. This gives further support to a RolC effect on GA biosynthesis rather than GA catabolism.

There is evidence that the GA_{19}-to-GA_{20} conversion is a major regulatory step in the biosynthesis of gibberellins in monocotyledons (Croker et al., 1990; Hedden and Croker, 1992) and is controlled by photoperiod in dicotyledons (Talon and Zeevaart, 1990; Olsen et al., 1995). Therefore, it is interesting that two distantly related dicotyledonous species such as tobacco and hybrid aspen both appear to respond to *rol*C expression by inhibiting the conversion of GA_{19} to GA_{20}. This strongly indicates that this is a key regulatory step in plants to reduce the synthesis of the active GA_1. Using hormone application studies, it has been demonstrated that the reduced GA_1 content in *rol*C transformants is a secondary effect, since 35S::*rol*C tobacco plants treated with exogenous GA_3 respond by increasing their internodal lengths, which in contrast to classical GA mutants, do not revert to a complete wt appearance (Schmülling et al., 1993; O. Nilsson, T. Moritz, B. Sundberg, G. Sandberg, and O. Olsson, unpublished data).

Since it has been suggested that the RolC protein is involved in cytokinin metabolism and, more specifically, in the conversion of inactive cytokinin glucoside conjugates to active free cytokinins (Estruch et al., 1991), we made a thorough characterization of the cytokinin content in our 35S::*rol*C hybrid aspen plants, using high-resolution mass-spectrometric techniques for quantification. Previously, more limited cytokinin quantifications were performed with 35S::*rol*C tobacco and potato plants, using both immunoassays (Nilsson et al., 1993; Schmülling et al., 1993) and LC-MS with internal standards (Nilsson et al., 1993). However, in contrast to those earlier studies, we measured not only members of all three major families of cytokinins (the Z, DHZ, and iP families), but also the two major conjugate forms, the *N*- and *O*-glucosides, thus obtaining a more complete picture of the cytokinin status of *rol*C-expressing plants.

Our data (Fig. 7) show that compared with wt plants, 35S::*rol*C plants have a dramatically increased content of ZR, especially in the shoot apical region. However, this was not linked to a decrease in the content of the major glucosidic conjugates; instead, these were also significantly increased. An earlier study showed that the principal conjugates formed after the application of Z and ZR to poplar are *O*-glucosides (Duke et al., 1979). The importance of these conjugates in *Populus* is further supported here, since both of the major *N*-conjugates Z7G and Z9G were below the level of detection, whereas the *O*-glucosides ZOG and ZROG were present at significantly higher levels. It seems unlikely that the increase in the ZR content that was found in *rol*C-expressing plants is due to RolC β-glucosidase activity increasing the hydrolysis of ZROG (Estruch et al., 1991), since the level of this compound was also significantly increased in our plants. Rather, our data support the model that *rol*C expression leads to an increased synthesis of Z-family cytokinins, which in turn induces conjugation to *O*-glucosides. To our knowledge, this is the first quantification of both of the proposed RolC substrates, the *N*- and *O*-glucosides, in plants expressing the *rol*C gene, and the data do not support the hypothesis that RolC exerts its action by hydrolyzing these conjugates to free cytokinins.

Increased cytokinin levels have been found also in 35S::*rol*C potato (Schmülling et al., 1993), another species that shows a strong phenotypic response to *rol*C expression (Fladung, 1990). On the other hand, the upper leaves of 35S::*rol*C tobacco cv W38, a species that responds less strongly to *rol*C expression, contained relatively low levels of both free and conjugated cytokinins (Nilsson et al., 1993), which implies reduced cytokinin biosynthesis. It should be noted, however, that the major decrease was found in the concentration of iPA, a cytokinin that was decreased also in 35S::*rol*C hybrid aspen (Fig. 7B). In tobacco, the concentration of ZR was not significantly altered and Z was slightly increased. Furthermore, the *N*-glucoside Z7G was the only conjugate measured and it showed a slight decrease (Nilsson et al., 1993). It is clear, however, that cytokinin quantifications in *rol*C-expressing tobacco would give a more complete picture if the *O*-glucosides are also included.

In conclusion, it seems plausible that the decreased IAA / ZR ratio in the shoot apical region of the *rol*C-expressing hybrid aspen plants would be sufficient to provoke many of the phenotypical alterations that are observed, such as a changed pattern of apical dominance, increased size of the apical meristem, and enhanced leaf production. These developmental changes will in turn certainly affect the metabolism of all plant hormones, leading to a complicated interplay between hormonal and developmental changes.

It cannot be excluded, however, that the altered cytokinin metabolism is a secondary effect. Although *rol*C has long been suggested to increase cytokinin activity, it has also been noted that the most biologically relevant *rol*C effect, namely root initiation, is strongly inhibited by cytokinin (Schmülling et al., 1988). The simplest explanation for these contradictory observations is that RolC does not primarily affect cytokinin metabolism. Therefore, the substrate for the published RolC β-glucosidase activity (Estruch et al., 1991) in vivo is not cytokinin glucosides, but another substrate with an important physiological action that, depending on the plant tissue and the developmental status, in turn affects cytokinin metabolism. Further studies on *rol*C, both in vivo and in vitro, coupled with the knowledge about the natural *A. rhizogenes* infection process, will shed new light on the nature of this enzymatic activity and thus provide important insights about the regulation of plant growth and development.

NOTE ADDED IN PROOF

Recently, Faiss et al., (1996) quantified both cytokinin *N*- and *O*-glucosides in tetracycline-induced 35S::*rol*C tobacco

plants, and concluded, as we did here, that RolC does not hydrolyze endogenous cytokinin glucosides in planta.

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